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QUALITATIVE METHOD FOR MEASURING OSTEOCLASTIC ACTIVITY

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INTRODUCTION

(A) *Why measure osteoclastic activity?* The cause of numerous poorly understood skeletal conditions, such as the various osteoporoses,[7,10] lies in a disturbance in the rates at which new bone is formed and at which old bone is resorbed. New bone formation is the product of osteoblastic activity. We have dealt elsewhere with its measurement by several different methods.[3,5,11,12,14]

Bone resorption is the product of osteoclastic activity. If osteoclastic activity is abnormal in a given disease, a cause must exist to produce the abnormality.[17] In order to detect aberrations in rate of osteoclastic activity, and in order to study the cause of such aberrations, a method for measuring osteoclastic activity must be available which is accurate and reliable.[17]

Such a method has not previously existed. In this paper we present a histological method possessing the requisite accuracy and dependability.

(B) *What is osteoclastic activity?* Living human bone, once formed, does not remain inertly in the skeleton for the remainder of the individual's life. After a certain period of time, on the order of 10 years in adults,[5] a given moiety of bone will be destroyed in vivo and replaced by new bone. The destruction is produced by cells termed osteoclasts which usually are seen in histological sections as multinucleated giant cells (Figure 1).

Osteoclastic activity must therefore be the sum total of the destructive activity of these cells.

(C) *Parameter of measurement of osteoclastic activity.* It would be desirable to measure the *volume* of bone being resorbed per unit volume of bone per unit time period. This would be a true, quantitative measurement of osteoclastic activity. In conjunction with the quantitative measurements of human osteoblastic activity previously published by the laboratory[5,12,14] such a measurement would permit an accurate statement of skeletal turnover and skeletal balance to be made.[5,12]

Unfortunately the measurement of osteoclastic activity cannot be done in this quantitative manner at present. A qualitative method of measurement must do for the time being. It will be a later task of the laboratory to "calibrate" the measurements

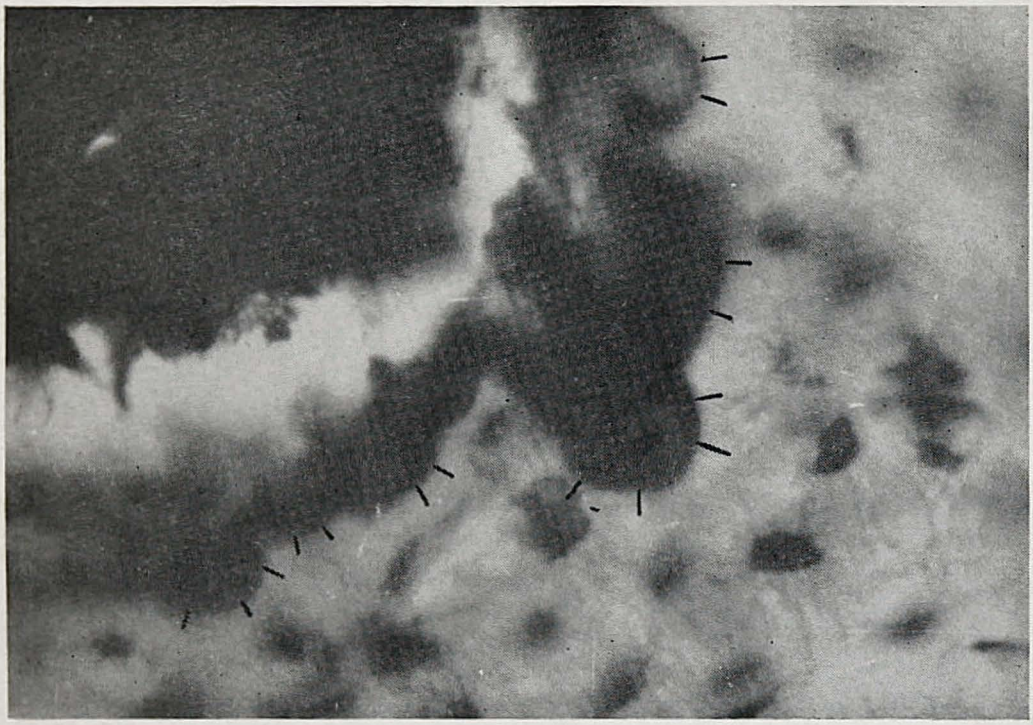

Figure 1

H & E, thick section, Villanueva's method, about 1000x. Osteoclasts are identified by the India ink marks. Osteoclasts are usually mobile, multinucleated, very active giant cells. They destroy as much bone in an hour as is formed by over 1000 osteoblasts in the same time period. The scalloped concavities left behind as the result of osteoclastic activity are known as Howship's lacunae.

so that they may be converted to or equated to true quantitative measurements of bone resorptive activity.

The parameter of measurement selected is the number of square millimeters of Howship's lacunae found on all the free bone surfaces in an average mm^3 of diaphyseal cortex. Howship's lacunae are the scalloped excavations left behind by osteoclasts (Figure 2). By free bone surfaces we mean the walls of Haversian and other vascular canals, the surfaces of trabeculae and the endosteal surfaces of bone.

This simple parameter of measurement had to be empirically proven useful (it has been). It contains certain theoretical objections which will be discussed later in this paper and which must be accounted for in interpreting the measurements.

Materials

The method is most suitable for cortical bone from any level in the diaphysis. The method may also be applied to cancellous bone by a modification which will be described. Human bones from the stapes to the femur, and long bones from rats, dogs, chickens, and frogs have been measured.

Material should be preserved in the undecalcified state in non-acidic media. Apart from these provisions, any current method of bone storage may be used.

Figure 2

Howship's lacunae. Undecalcified cross section human clavicle, 50 microns thick, basic fuchsin. About 300x N.A. 0.65. The lacunae are scalloped. They are approximately as deep along the perpendicular to the page surface as they are wide. Their average width is 35 microns.

If other types of measurements, or other features in addition to osteoclastic activity, are to be measured, the sections should be prepared fresh from fresh, undecalcified bone. Although it is not widely known, considerable artifact results in fresh, undecalcified bone from storage in the deep freeze, in formaldehyde, in ethanol or from simply drying of the bone prior to preparation of sections.[13] A number of publications have appeared recently dealing with work done by other investigators and based on undecalcified sections of bone. Unfortunately these publications are accompanied by some illustrations revealing artifact from improper storage; worse, they are accompanied by some erroneous interpretations based on these artifacts.

METHODS

(1) SECTIONS — Undecalcified, complete cross sections of the bones to be measured are made by Frost's method.[4] These are stained with basic fuchsin and mounted in synthetic resin mountant.[6] Final section thickness should be about 50 microns. Reasonable accuracy in the perpendicularity of the cut to the long axis of the diaphysis should be ensured.

Incomplete cross sections and longitudinal sections are not suitable for the present measurements. The reason is the "longitudinal homogeneity, transverse inhomogeneity" which seems to characterize the distribution of a number of measurable features in human bone, [8,9,11] among them being osteoid seams, micropetrosis, feathering — and osteoclastic activity.

(2) HOWSHIP'S LACUNAE — These lacunae are easily recognized. They consist of concavities in the free surface of the bone about 35 microns in width. The depth of the excavation is 0.1 to 0.5 in width. (Figure 2). Howship's lacunae often appear only on part of the wall of an Haversian canal, on part of the surface of a trabecula or endosteal marrow space, or only on part of the wall of a resorption space within the cortex. This irregularity in distribution affects not only the circumference of these structures (the X and Y axes of

polar coordinate space), (Figure 3), but also the vertical axis which corresponds to the optical axis of the microscope (or Z axis of polar coordinate space). *The method of detecting and measuring Howship's lacunae must be able to distinguish irregular distribution in the Z axis as well as in the X-Y axes of the section.* This requirement is not met on microradiographs due to the great depth of field of the x-ray beam.

In the routine, decalcified, microtome cut, 7 micron thick sections common in pathological and histological practice, the recognition of Howship's lacunae by an *experienced observer* is only about 70 percent reliable. In stained, undecalcified sections more than 30 microns thick the reliability of recognition of Howship's lacunae is on the order of 98 percent. The major reasons for this difference seem to be the presence of increased depth perception in the thicker sections at moderate apertures which results from accommodative ability in the eye, plus the relative lack of shrinkage and distortion in undecalcified sections (Table I).

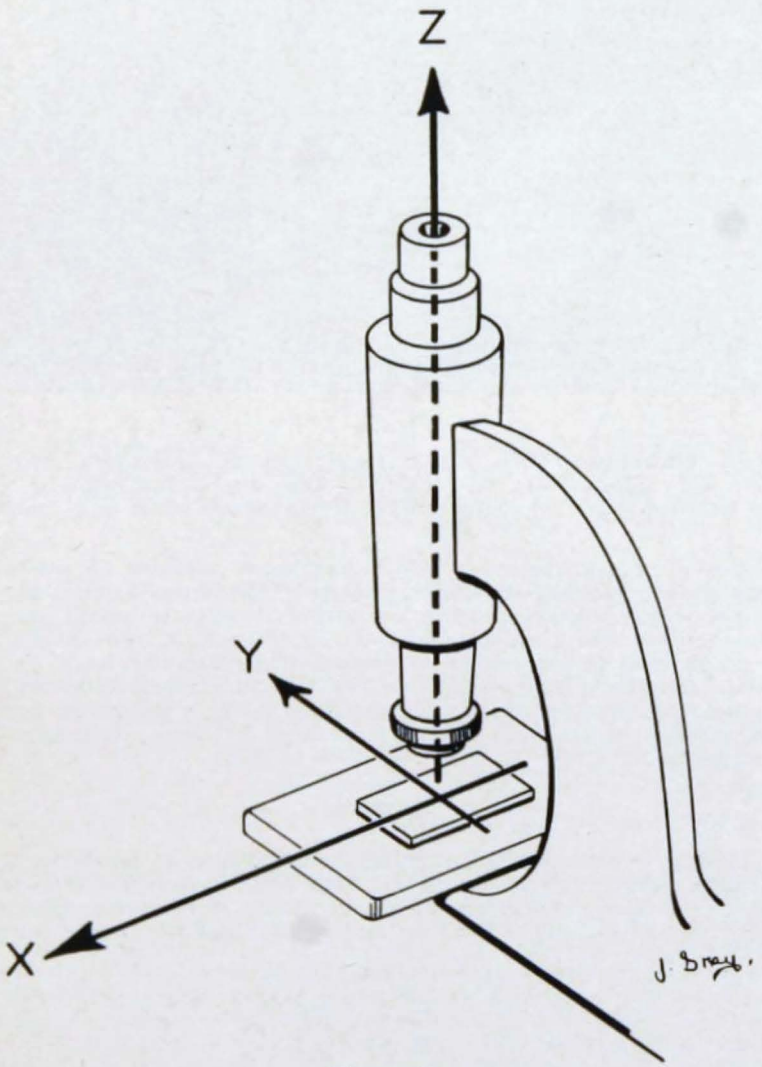

Figure 3

Illustration of the (X), (Y), and (Z) axes of polar coordinate space. In terms of the microscope and microscope slide, the (X) axis is the length of the slide, or east-west direction. The (Y) axis is the width, or north-south direction. The observer looks along the (Z) axis when he looks through the eyepiece.

(3) OPTICAL SECTIONING — In theory, given a solid composed of two different phases which are well mixed, accurate measurement of the proportions of the two phases may be made on an infinitely thin plane cut through the solid. In theory this plane will have the same proportion of its surface area composed of phase (A) as the proportion of phase (A) is in the whole volume of the solid. Also in theory, the total length of the interfaces between phase (A) and phase (B) in the theoretical plane will be proportional to the total surface area of the interfaces in the whole solid.[15] The crucial assumptions in this theory are that mixing of the phases is homogeneous and the plane cut through the solid is large enough so that random distributional effects are insignificant.

Optical sectioning is the technique of using a microscope objective to provide an optical plane through a transparent specimen. This optical plane represents the theoretical infinitely thin plane previously referred to and is the image formed by the objective. Measurements made on the image formed by the objective are valid provided certain limitations are known and compensated.[1,2,16]

All microscope objectives have some depth of focus. Depth of focus is the distance along the optical axis (Z axis) within which an object appears to be in sharp focus. Depth of focus is large in objectives with small numerical aperture (N.A.) and small in objectives with N.A. in excess of 1.2. The depth of focus in microscope objectives is analogous to the depth of focus in camera lenses.[1,2,16]

The relation between the depth of focus of the objective and the linear depth along the Z axis of the structure which is to be sampled and measured by optical sectioning determines the accuracy of the method, assuming only that the dimensions of structure being measured are much larger than the wave length of the light being used. Specifically, in the present case, the average depth of Howship's lacunae is about 35 microns. An objective with a depth of focus of ten microns will accordingly introduce error in measuring Howship's lacunae of about 30 percent based on optical sectioning, while with a depth of focus of 1.0 micron, the error is about 3 percent. In both cases the measurement will be too large by the amount noted. Once known, the measurements can be corrected for this error. There is such individual variation in visual acuity and in cortical visual integration that the individual investigator should preferably determine his own error.

Table I

Objective Tested	A	B	C	D	E	F	G	H	I	J	K	L
Objective N. A.	.16	.20	.20	.32	.32	.40	.63	.63	.65	.65	.75	.95
Objective Magnification	6.3	6.3	8.0	10.0	16.0	16.0	25.	40.	40.	40.	40.	40.
Type of Objective	Ach	Fl	Ach	Apo	Ach	Fl	Apo	Ach	Ach	Ach	Fl	Apo
Ach: achromat Apo: apochromat Fl: fluorite Eyepiece Magnification	8.	8.	8.	8.	8.	8.	8.	8.	8.	8.	8.	8.
Depth of Field, microns	61.3	37.7	23.7	20.1	6.7	7.6	3.8	1.9	1.7	2.0	1.6	0.9
Correction Factor: See Methods (4)	220.%	137.%	86.%	73%	24.4%	27.6%	13.8%	7%	6%	7.3%	5.8%	3.3%

Depth of focus (visual) determined by senior author (H.M.F.) on a series of Zeiss objectives 160 mm tube length, 8x compensating eyepiece. Fine motion Zeiss WL stand used; objective focused on dirt specks in mount of barely visible dimensions. Brightfield, Kohler illumination, fully illuminated aperture, achromatic-aplanatic condenser. Each value is the average of seven determinations in brightfield. Increasing eyepiece magnification would diminish the visual depth of field within limits. Number 1 cover slip; H.S.R. resin mountant. The correction factor is calculated by the formula 1.27 T/D where T is the depth of focus of the objective-eyepiece combination and D is the average width of a Howship's lacuna (35 microns). The formula is approximate rather than exact. It should be noted that visual depth of field includes the accommodative capacity of the observer's eye. This is included because it is present during the measuring act.

The approximate depth of focus of objectives with varying N. A. is given in Table I. These values are accompanied by the approximate error introduced into the present measurements by this depth of focus. The error will be different for structures with different dimensions along the optical or Z axis of polar coordinate space. The error varies with variation in tube length or eyepiece magnification.

Since the depth of focus of the microradiographic beam is greater than any conceivable thickness of section which might be measured in the type of work under consideration, the amount of error introduced in measuring osteoclastic activity in microradiographs is in the hundreds of per cent and difficult to compensate.

(4) CORRECTION FACTOR — The actual measured value of Howship's lacunae is corrected for the error caused by the finite depth of focus of the objective by the simple, approximate formula:

$$A = M\left\{\frac{100}{100 + x}\right\}$$

where (A) is the correct value for square millimeters of lacunae per cubic millimeter of bone, (M) is the measured value and (X) is simultaneously a correction factor and is the percentage error introduced by the numerical aperture of the objective used (Table I).

(5) SELECTION OF OBJECTIVE — In theory all consideration of error due to finite depth of focus of objectives could be eliminated by performing the measurements with an oil immersion objective having an aperture over 1.2. The error introduced would be less than 1 percent and could be ignored. In practice the large initial magnification of such objectives would require measuring so many separate fields on one section to obtain an adequate and representative sample that no systematic program of measurement would be feasible or endurable.

As a compromise of the wish to obtain as little depth of field as practical while not unduly multiplying the number of fields to be measured in any one specimen, we selected an achromat of N. A. O.65, 45X. Depth of focus of this objective with 8X eyepieces is about 2.0 microns. The error introduced by this depth of focus is about 7 percent and is compensated in individual cases by formula in section (4) preceding.

(6) EYEPIECE — The magnification of the eyepiece plays its part in the above error. The eyepiece should be selected to enlarge the optical limitations inherent in the microscope in excess of any resolving power limitations present in the investigator's eye, by reason of uncorrected ocular aberration (such as astigmatism).

The power of the ocular used should otherwise be as low as is compatible with the above qualification.

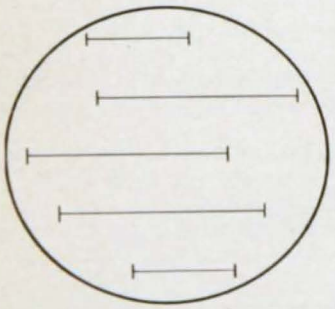

Figure 4

Diagram of the reticule in the Zeiss Integrating Micrometer Eyepiece II. Each of the middle horizontal lines is 0.2 the total combined length of the lines. Each of the top or bottom lines is 0.1 the total combined length of the lines. The vertical spacing is equal.

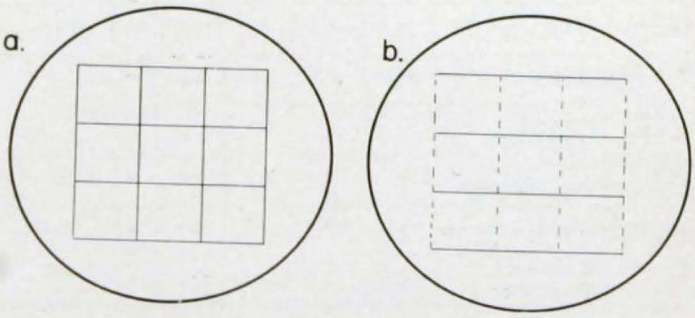

Figure 5

A typical net micrometer reticule as it would appear in place in a micrometer eyepiece. By considering only the horizontal lines, and ignoring the vertical lines as indicated in Figure 5B, the same purpose may be served as by the more elegant Zeiss eyepiece.

The ocular must contain a reticule. The Zeiss Integrating Eye-piece Micrometer II is designed for measurements such as the present ones and if available, it is recommended[18] (Figure 4). A satisfactory substitute is a net micrometer reticule inserted in a micrometer eyepiece of 7X or 8X magnification [15] (Figure 5). Both reticule and eyepiece may be purchased from any of the major microscope manufacturers.[1,2,16]

Whichever eyepiece is utilized, when it is in place in the eye tube of the microscope, and a section to be measured is in focus under the objective to be used in the measurements, the lines of the reticule will appear superimposed on the image from the objective. Focusing the eye lens of the micrometer eyepiece will sharpen the image of the reticule. The eyepiece should be rotated so that one set of lines in the reticule is horizontal (parallel to the X axis).

(7) CALIBRATION OF THE EYEPIECE — With a metric stage micrometer, the total length of all of the horizontal lines in the eyepiece reticule is determined and recorded as (L) for future use.[1,16] In the Zeiss integrating eyepiece this procedure is simpliified somewhat according to the instructions that accompany each eyepiece.[18]

This calibration is good only for the combination of objective and eyepiece used. If different objectives are used, a separate calibration for each one must be made and recorded for future use in calculations.

(8) MEASUREMENT PROCEDURE — The horizontal lines of the eyepiece serve as a means of sampling the image formed by the microscope objective. This image in turn represents the theoretical, infinitely thin plane cut through the bone section to be measured. Because the finite depth of field of the objective is a small fraction of the actual depth of the Howship's lacunae to be measured, the procedure is feasible.

With a section to be measured in place under the objective and focussed correctly, the spaces normally found in bone may be seen. These spaces, of present interest, are the various vascular channels. Superimposed on the image of the section will be the lines engraved on the eyepiece reticule (Figure 6).

Each point where a line of the reticule cuts across a Howship's lacuna that is in sharp focus is recorded as a "hit" with a tally. Each field measured is recorded on a separate tally. A new field is considered counted each time the eyepiece is rotated and a new field is counted each time the setting of the fine adjustment of the microscope is changed. It is important that during the inspection of any single field, the fine motion not be changed and the eyepiece not be rotated.

The accuracy of measurement of any single area of the section encompassed by one field can be increased by the simple device of rotating the eyepiece as many separate times, and altering the fine adjustment as many separate times, as is required. Each change is counted as a new field and the hits are also counted.

The total number of hits, and the total number of fields, must be separately and accurately recorded for subsequent calculation. Hits are identified as (H) and fields as (F).

(9) SAMPLING PROCEDURE — Given: a cross section of human femur. To measure the Howship's lacunae in the total section would require a formidable number of separate determinations. Sufficient accuracy may be had by applying a systematic method of sampling the section. This method must be such that any given area or any given quadrant of the section is represented in the final, totalled figures exactly by its fair share. Too many observations near the linea aspera, for example, will lead to artificially high numbers of hits because osteoclastic activity is higher here than over the average of the entire section.

The sampling method used is one we have previously published in measuring osteoid seams per cubic millimeter of cortex[11] (Figure 7).

Briefly, this sampling method involves measuring the section along the (X) axis with the aid of a mechanical stage on the microscope. After each field is measured, the section is moved along the (X) axis exactly the width of one field, the next measurement made. When one strip of the section has been scanned thus, the section is moved along the (Y) axis exactly one field diameter and this new strip similarly measured. The entire section is scanned in this manner. In large sections every odd field or strip skipped. When the periosteal or endosteal cortex enters the field of view, the affected field is not measured; instead a new scan along the (X) axis is begun, beginning with the periosteal or endosteal surface positioned exactly at the periphery of the field of view.

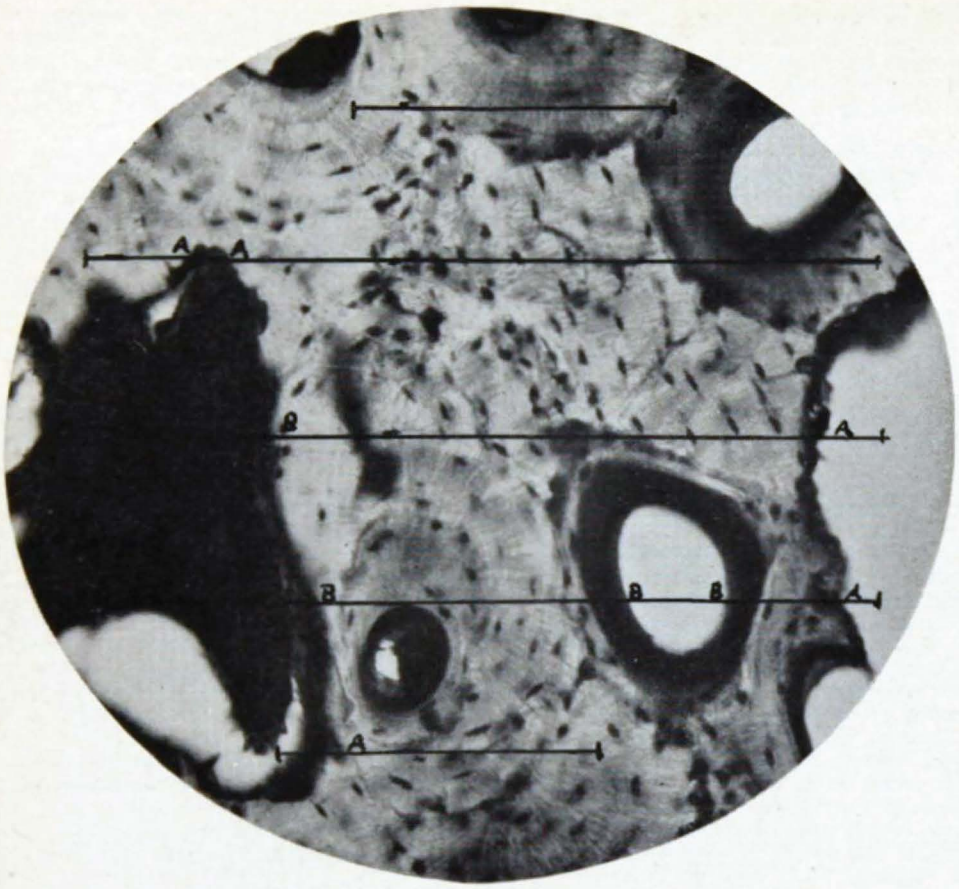

Figure 6

The reticule of the Zeiss Integrating Eyepiece II is superimposed on a microscopic field. "Hits" on Howship's lacunae (A) and hits on surfaces not covered with Howship's lacunae (B) are indicated. By rotating the eyepiece or by changing the setting of the fine motion or by moving the slide a new field with its own set of hits is measured. This is basically a point counting technique. Precision requires that the results of a large number of measured fields be averaged. Precision requires a uniform and properly designed sampling technique.

When the cortex of the bone to be measured is thin, objective and eyepieces may have to be selected with a view to obtaining sufficient magnification to permit the sampling technique outlined. Such new optical combinations require separate micrometer eyepiece calibration and separate determination of correction factor due to depth of field.

As in measurements of osteoid seams, the number of sections which should be measured to obtain workable accuracy varies according to the absolute size of the section. Only one human femoral cross section need be measured for workable precision, while three or four ribs and perhaps five rat femurs must be measured because the total cross section area of of these sections diminishes progressively. The decrease in total cross section area is accompanied by an increase in the importance of random distributional error in any one section.

(10) CALCULATION of mm^2 OF HOWSHIP'S LACUNAE — At the conclusion of the measuring procedure there are three figures on hand: (L) = the total length of the horizontal lines in the eyepiece reticule; (H) = the total hits; (L) = total fields.

First calculate the average number of hits per field (H_{pf}):

$$H_{pf} = \frac{H}{F}$$

Second, calculate the measured mm^2/mm^3 Howship's lacunae (A):

$$A = 2\frac{H_{pf}}{L}$$

The mathematical reasoning behind this maneuver is outlined fully in references[15,18] The procedure is well established and is valid for the present purpose as long as the technique recommended is adhered to. A typical value for human rib is 0.30 mm^2/mm^3.

(11) MEASURING THE PERCENTAGE OF FREE SURFACE COVERED WITH HOWSHIP'S LACUNAE — In measuring cancellous bone, and for certain other purposes, it may be desirable to express the measurements in terms of the percentage of the free bone surfaces which are covered with Howship's lacunae. This is simpler than the foregoing procedure and does not involve as much calculation.

During the measuring procedure, simply record the number of hits on Howship's lacunae on one tally and the number of hits on free surfaces not containing Howship's lacunae with a separate tally. The total number of fields need not be recorded, nor need the reticule be calibrated, for such measurements.

If absolute values for both types of surface are required — and we recommend this procedure, which we follow — then a third tally is used. One tally keeps track of the total fields, the second the line intersections with Howship's lacunae and the third the line intersections with free surfaces not containing Howship's lacunae. Calculations are done as outlined in (10) above.

It must be rememberd that the *total* surface is represented by the sum of Howship's and non-Howship's hits. The percentage of either in relation to the whole must be determined by adding the two together to obtain the whole, and the dividing the desired value by the total.

Example: In a particular section the Howship's mm^2/mm^3 measures 0.10; the non-Howship's measures 2.3; the total surface is 2.4 so that the Howship's is 0.10/2.4 or 4.2% (a high normal value). The formula for this procedure which is the recommended one is:

$$P = \frac{H}{H + N}$$

where (P) is the percentage of free surface covered by Howship's lacunae, (H) is the number of hits on Howship's lacunae and (N) the number of hits on non-Howship's lacunae surfaces.

Example: In a particular section the hits on Howship's lacunae number 100; the hits on surfaces not covered by Howship's lacunae number 900. The total is 1000. Howship's lacunae are 100/1000 or 10 percent of the total free surface area (an elevated value).

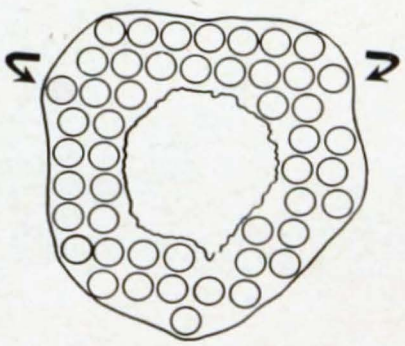

Figure 7

Sketch of cross section of a human femur. The small circles superimposed represent the fields visible with the combination of objective and eyepiece used for these measurements. The section is scanned in strips, using the orthogonal motions of a mechanical stage to ensure uniformity. The fields measured are separated from each other by exactly one field diameter. The horizontal strips are one field diamter wide. In small sections the entire section must be scanned, depending on the required precision and the available patience.

Discussion

Several problems must be considered which are inherent in the nature of osteoclastic physiology.

(A) Some authorities still feel that dissolution of bone in vivo may occur by some mysterious means other than osteoclasia. If this were true the present method would be useless. We believe, however, that there is no longer any reasonable basis for such a belief. Extensive experience with normal and abnormal human and animal material, (including situations reported by some authorities as examples of nonosteoclastic resorption of bone) convince us that the osteoclast is the only route to *in vivo* bone resorption.

(B) In the normal course of events a Howship's lacuna is covered by new bone (Figure 8). The length of time during which a Howship's lacuna remains exposed on a free surface is thus dependent on the rate of formation of new bone. Accordingly variations in osteoblastic activity may produce differences in the *measurement* of osteoclastic activity performed by the present method, even though in different cases the real osteoclastic *activities* were identical. On the other hand, widely differing

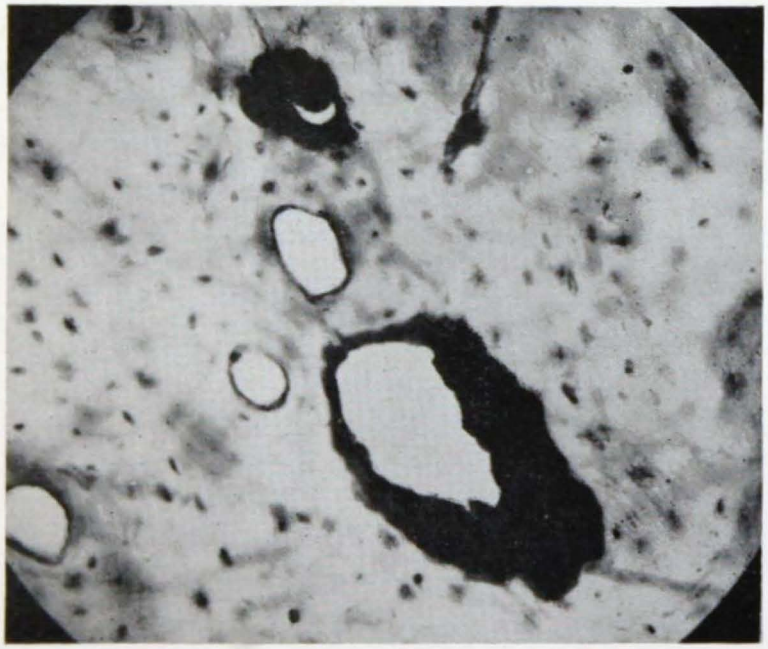

Figure 8

Undecalcified cross section human tibia, about 100x, stained with boiling 0.1% $KMnO_4$. Just at 4 o'clock of center is a resorption space being filled in by a new Haversian system. The black, meniscus shaped layer is the new bone, densely stained by MnO_2. The periphery of this layer reveals the characteristic scalloping of osteoclastic activity. Osteoclasts formed the tunnel, then disappeared. Then new bone was laid down on the walls of the resorption space. In its present form, therefore, no Howship's lacunae would be recorded because they are no longer exposed on free surface. Some weeks prior to the time of sampling, the Howship's lacunae would have been exposed and tallied. The quickness with which the new bone covers osteoclased surfaces, therefore, must affect the osteoclastic *measurements*, although it is independent of the true osteoclastic *activity*.

osteoclastic activities might be found to measure identically provided there existed an alteration of osteoblastic activity of similar sign and degree in each instance.

The result of these facts is that osteoblastic activity must be measured as well as osteoclastic activity if meaningful comparisons between different bones or cases or diseases are to be made. More than this, the relative effect on measured osteoclastic activity of specific variations in osteoblastic activity must somehow be determined before reliable interpretation of osteoclastic measurements can be done.

(C) The measurement of osteoclastic activity obtained, even after the corrections pointed out in (B) are applied, provide figures of relative significance only. *It will be necessary to convert or equate these figures to a specific amount of bone resorbed in a unit volume of the skeleton in unit time.* Only then will a truly quantitative measure of osteoclastic be available. This "calibration" of the method we report here will be dealt with in a separate publication; it can be accomplished by indirect means with a satisfactory degree of accuracy.

(D) While it is possible that individual variation in single patients may prevent drawing any usefully significant conclusions about osteoclastic activity in the single case for the present, knowledge of normal values in varying age groups, in the two sexes and in various diseases may be gained by the device of averaging the results of sufficiently large numbers of similar cases. This device permitted us to measure the effect of age on human osteoblastic activity.[5,12,14] Once group norms are known, attention may be devoted to obtaining information that is useful in the single case, and therefore useful in diagnostic clinical practice.

In conclusion, we feel that the existence of feasible methods of measuring human osteoblastic and osteoclastic activity paves the way for the beginning of a new level of understanding of the factors affecting these activities. Being measurable, they may be observed with confidence. Being observable, it should become possible to understand them and the factors that govern them. When the governing factors are known and understood, we may become able to manipulate them. Gaining this control is the objective of the work of this laboratory.

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